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Valorization of sugarcane bagasse and sugar beet molasses using *Kluyveromyces marxianus* for producing value-added aroma compounds via solid-state fermentation

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Abstract

The growing demand for natural products has favored the development of bioprocesses for obtaining value-added products in the fragrance and flavor sector. Solid-state fermentation (SSF) of agro-industrial residues together with generally recognized as safe (GRAS) strains like *Kluyveromyces marxianus* appears as a remarkable alternative for producing aroma compounds. In this study, a continuous air supplied system was used for optimizing the production of fruit-like compounds via SSF of a mixture of sugarcane bagasse/sugar beet molasses employing *K. marxianus*. The main operational parameters were evaluated to identify the best conditions for their biosynthesis. Maximum cumulative volatile production was achieved at 40°C, 35% molasses (dry basis) and specific air flow rate (S_{AFR}) 0.14 L $h^{-1}g^{-1}_{ITS}$ reaching 161 mg_{TVOL} per gram of initial total solids content (ITS). The main components of the produced volatiles were alcohols (43%) while ester species constituted only 18%. Selectivity to ester species was enhanced working at 30°C, 25% molasses and S_{AFR} 0.11 L $h^{-1}g^{-1}_{ITS}$, maximizing the ester content to 47.6 mg_{Ester} g^{-1}_{ITS}. In this case, a pleasant fruity odor was perceived thanks to the higher ester content (35%). Stressing conditions for *K. marxianus* like high temperatures and limiting nitrogen availability seem to be key factors for promoting the production of the volatile compounds. *

*Abbreviations

AFP: Air filled porosity [% mL mL^{-1}]
CO_2^p: Carbon dioxide production rate [mg_{CO2} kg^{-1}_{vs} h^{-1}]
CFU: Colony forming units
GRAS: Generally recognized as safe
ITS: Initial total solids content
K.M.: *Kluyveromyces marxianus*
MAC: Maximum adsorption capacity [% g_{H20} g^{-1}_{sam}]
OUR: Oxygen uptake rate [mg_{O2} kg^{-1}_{vs} h^{-1}]
RQ: Respiration quotient
SSF: Solid-state fermentation
S_{AFR}: Specific air flow rate [L h^{-1} g^{-1}_{ITS}]
SBM: Sugar beet molasses
SCB: Sugarcane bagasse
TD: Thermal desorption
T_{vol}^{Acc}: Total cumulative volatile production [mg_{Tvol} g^{-1}_{ITS}]
$T_{vol,t}$: Total volatile productivity [mg_{Tvol} h^{-1} g^{-1}_{ITS}]

Keywords

Solid-state fermentation, *Kluyveromyces marxianus*, Sugarcane bagasse, Fruit-like compounds, Agro-industrial residues.

1. Introduction

Aroma compounds play a major role in a great variety of applications like food, cosmetic, chemical and pharmaceutical industries by improving the organoleptic properties of the products. Currently, annual fragrance and flavor (F&F) market is estimated in US$26.5 billion with increases of almost 4% each year (The freedonia group, 2015). This represents over a quarter of the world market for food additives of which 13% share correspond to aroma compounds (Leffingwell & Associates, 2015). Typically, they are obtained by extraction from the matrix that contains them, but their low concentration makes the recovery process costly. Conversely, synthetic flavors have been produced by chemical routes, but these substitutes just partially reproduce an aroma. As a consequence, despite the lower cost of synthetic aromas, their quality is far away from their natural counterparts (Dastager, 2009). Furthermore, the rising demand of consumers for natural products poses in evidence the need for alternative processes. A promising substitute route for aroma production is based on microbial biosynthesis or bioconversion (Ben Akacha and Gargouri, 2015; Longo and Sanroman, 2006; Vandamme and Wim, 2002). These bioprocesses based on microorganisms and enzymes involve the synthesis of flavors as secondary metabolites during fermentation of nutrients such as sugars and amino acids. These processes are also encouraged by the current legislation based on their qualification of Generally Recognized As Safe (GRAS) products by entities like the Food and Drug Administration (FDA) (Dubal et al., 2008; Medeiros et al., 2010), considering natural aromas those including biotechnology-derived products based on microorganisms, plant cell cultures and enzymes.

In addition, bioconversion for producing aroma compounds is in accordance with the aim of exploiting renewable sources and agro-industrial wastes to promote clean production, less energy intensive systems and more economical processes (Laufenberg et al., 2003). Particularly, agro-industrial residues represent a good choice as substrates for these microbial biosyntheses since they are rich in carbohydrates and other nutrients (Sarma et al., 2014). One of the largest agro-industrial by-products worldwide is the sugarcane bagasse (SCB), a fibrous leftover obtained after the extraction of the juice contained in the sugarcane. SCB is commonly used for energy production in the same sugar industry, but more recently it has been used as source for several biotechnological applications (Pandey et al., 2000). On the other hand, solid-state fermentation (SSF) has been proved as a prominent alternative for the processing of several solid wastes to perform their transformation into value-added products (Farinas, 2015). SSF is a versatile process able to produce a wide range of products like enzymes (Abraham et al., 2014; Cerda et al., 2016), biopesticides (Ballardo et al., 2016), biosurfactants (Jiménez-Peñalver et al., 2016), and biofuels (Li et al., 2013).

SCB has been tested in the production of aroma compounds by SSF in the production of coconut-like aroma using *Trichoderma* strains (Fadel et al., 2015; Ladeira et al., 2010) and also for producing fruity aromas by means of *Ceratocystis fimbriata* (Christen et al., 1997). Nevertheless, some other strains have also proven their efficiency in the production of aroma compounds in SSF like *Rhizopus oryzae* (Bramorski et al., 1998a), *Phanerochaete chrysosporium* (dos Santos Barbosa et al., 2008), *Saccharomyces cerevisiae* (Mantzouridou et al., 2015) and *Kluyveromyces marxianus* (Aggelopoulos et al., 2014; Medeiros et al., 2001).

115 Among these, *K. marxianus* appears as one of the most promising strains given its
116 versatility, capability to grow in different solid media and under extreme conditions,
117 producing a broad diversity of secondary metabolites efficiently (Fonseca et al.,
118 2008; Lane and Morrissey, 2010). Although those studies show the feasibility of
119 producing fruit-like compounds via solid-state fermentation by means of several
120 strains, there are some challenges that must be addressed in order to use it in
121 large-scale processes. Some of them include the use of only residues as substrates,
122 understanding the behavior of the process on a bigger scale (including the particle
123 size distribution, type of aeration) and comprehending the effect of the operating
124 parameters in the type of volatiles produced (Selectivity to fruit-like compounds) as
125 well as their total productivity.
126 The aim of this work was the optimization of the SSF process for producing fruit-like
127 compounds using a mixture of an agro-industrial residue (sugarcane bagasse) with
128 an industrial by-product (sugar beet molasses) after inoculation of the GRAS strain
129 *K. marxianus*. For this purpose, a continuous air supplied system was used (0.5 L,
130 90 g substrate capacity). The specific objectives include: (i) to optimize the
131 productivity of total volatiles and ester species in terms of the substrate composition,
132 temperature and aeration rate; (ii) to determine the effect of MC and C:N ratio on the
133 selectivity and productivity of the volatile compounds in the particular mixture used
134 as substrate; (iii) to go more deeply into the cause-effect relationship between
135 selectivity of volatiles and the monitoring variables.
136

137 **2. Materials and methods**

138 2.1. Microorganism

Kluyveromyces marxianus (ATCC 10022) was obtained from *Colección Española de Cultivos Tipo* (CECT, Valencia, Spain) and it was grown for 24 h at 30°C on agar slants containing 40 g L^{-1} glucose, 5 g L^{-1} yeast extract, 5 g L^{-1} soy peptone and 20 g L^{-1} agar. The strain was preserved at -80°C in cryovials containing impregnated pearls with *K. marxianus* The inoculum was prepared adding one pearl to a 250 mL Erlenmeyer flask with 100 mL of liquid medium consisting of 40 g L^{-1} glucose, 5 g L^{-1} yeast extract and 5 g L^{-1} soy peptone. Afterwards, the culture was incubated on a rotary shaker at 30°C and 150 rpm for 20 h. All media and materials were previously sterilized by autoclaving at 121°C for 20 min. Cells suspension was counted by serial dilutions prepared in NaCl 9 g L^{-1} and expressed as CFU (colony forming units) per mL of solution.

2.2. Substrate preparation and suitability

Sugarcane bagasse was first dried at 60°C in an air oven for 24 h. The dried substrate was ground in a granulator mill obtaining a particle size distribution between 0.5 and 32 mm (Figure S1). It was stored at -20°C until its preparation for the fermentation. Preparation of the dried sugarcane bagasse consisted in adjusting its moisture content, pH and molasses load. It was performed by means of a 1:1 (v:v) mixture of a phosphate buffer pH 7 (0.1M) and a nutrient solution containing 1.5 g L^{-1} $Fe(NO_3)_3.9H_2O$, 0.8 g L^{-1} $ZnSO_4.7H_2O$, 0.4 g L^{-1} $MnSO_4.4H_20$, 3.0 g L^{-1} $MgSO_4.7H_2O$ and 1.9 g L^{-1} $(NH_4)_2SO_4$, which was prepared based on the results of (Christen et al., 1997). Molasses were added and dissolved in this mixture until the final amount corresponded to the requirements of the experiment (measured as a fraction of the total solids of the SCB). For the C:N ratio experiments, yeast extract

was used as nitrogen source, and it was added and dissolved in the aforementioned liquid mixture. Once substrate was impregnated, it was autoclaved at 121°C for 20 min and after cooling it was inoculated using approximately 10^8 CFU per gram of initial total solids content of substrate (g_{ITS}).

Although previous works (Medeiros et al., 2000) did not show a high production of aromas from these substrates, a preliminary screening of materials (data not shown) performed under the present SSF conditions (dynamic aeration, larger scale, particle size control) showed that sugarcane bagasse was a good starting waste for the production of aromas. Other works supported these findings (Christen et al., 1997).

2.3. SSF experiments

SSF was carried out in 500 mL Erlenmeyer flasks containing 90 g of the prepared substrate. Each experiment consisted of a triplicate placed in a temperature-controlled water bath and connected to a mass flow controller (Bronkhorst Hitec) that supplied continuously humidified air to each flask across the lid to the bottom such that the air is forced to flow through the substrate until it reaches the top of the reactor as described by (Jiménez-Peñalver et al., 2016; Ponsá et al., 2010). Gas streams were independently led to an oxygen sensor (αLphase Ltd.) in series with an IR CO_2 sensor (Sensotran IR), both connected to an on-line system (Indusoft Studio) that recorded O_2 and CO_2 concentrations. The respirometric analysis was performed using this information to compute the oxygen uptake rate (OUR) and the carbon dioxide production rate (CO_2^p) as stated by (Medeiros et al., 2001; Ponsá et al., 2010). The SSF experiments were followed during 72 h.

187

188 2.4. Analytical methods

189 2.4.1. Determination of volatile compounds in the gas phase by TD-GC-MS

190 The composition of the exhaust gases of the fermented substrate was determined
191 by thermal desorption gas chromatography mass spectrometry (TD-GC-MS). For
192 this purpose, a set of metallic sampling tubes containing 380 mg of Texan TA 35/60,
193 Carbograph 1TD 40/60 and Carboxen 1003 40/60 (Markes Int.) were used to
194 capture the volatile compounds after grabbing a 100 mL of headspace (outlet of the
195 reaction system) with an easy-VOC system (Markes Int.). Samples were stored
196 capped at ambient temperature for a maximum of 2 days until their analysis. TD-
197 GC-MS was performed in an Agilent 7820A coupled to 5975MSD and TD Unity2
198 (Markes Int.). Thermal desorption of sampling tubes was carried out in a two-step
199 mode. First, volatile compounds were desorbed at 280°C during 5 min and a flow
200 rate of 80 mL min^{-1} of He to be driven to a graphitized carbon trap at 2°C.
201 Afterwards, the trap was desorbed in a second step at 300°C during 3 min using fast
202 heating (100°C s^{-1}). The capillary transfer line to the GC was kept at 170°C, while
203 specific split flows were set to adjust the resolution of the chromatogram and to
204 avoid saturation of the column. All sampling tubes were conditioned at 330°C for 35
205 min after each measurement to avoid interferences.

206 Separation of the volatile compounds was performed in a capillary column DB-
207 624ms 60mx0.25mmx1.4µm. Injection port was kept at 220°C while oven
208 temperature was held at 40°C for 5 min, then raised till 90°C by 5°C min^{-1}, followed
209 by an increase until 200°C by 20°C min^{-1} and concluding with a rise till 240°C at
210 35°C min^{-1}. Electron impact mass spectra were recorded at 70eV ionization energy

in the 15–300 m/z mass range. Ion trap and GC/MSD interface temperatures were 150 and 230°C, respectively. The identification was carried out by comparing the retention times and mass spectra of volatile compounds to those of the selected analytical standards (Table 1) by mass spectra obtained from wiley275 library. Quantification of the individual components was performed using calibration curves (external standard) using analytical standards (sigma-Aldrich) and the calibration solution loading rig (CSLR) of the Unity system.

Total volatile productivity at time t, was computed using equation 1:

$$T_{vol_t} = \sum_{i=1}^{n} S_{AFR}\, C_i \qquad (1)$$

Where T_{volt} is the total volatile productivity in the gas phase at time t ($mg_{Tvol}\ h^{-1}\ g^{-1}{}_{ITS}$), S_{AFR} is the specific air flow rate supplied to the reactor ($L\ h^{-1}\ g^{-1}{}_{ITS}$), C_i is the headspace concentration of the compound *i* ($mg_i\ L^{-1}$), and *n* is the total amount of quantified volatile compounds. Similarly, the total cumulative volatile production was calculated through the numerical integration in time of productivity (until 72 h) as detailed in equation 2.

$$T_{vol^{Acc}} = \int_0^{72} T_{vol_t} dt \approx \frac{1}{2}\sum_{t=0}^{72}(T_{vol_t} + T_{vol_{t+1}})\Delta t \qquad (2)$$

Where $T_{vol}{}^{Acc}$ is the total cumulative volatile production ($mg_{TVOL}\ g^{-1}{}_{ITS}$).

Table 1.

235 2.4.2. *K. marxianus* enumeration

236 10 g of sample were transferred to a sterile bottle where 100 mL of NaCl 9 g L^{-1}
237 were added. The mixture was agitated for 10 min at 100 rpm in an orbital shaker at
238 room temperature. This solution was used as stock for making appropriate decimal
239 dilutions in NaCl 9 g L^{-1} and plated on Petri dishes (triplicates) containing the same
240 medium used for the growing of the pure strain. Cultures were incubated for 24 h at
241 30°C, and the population was determined by counting the units in the Petri dishes.
242 Results were expressed in CFU g^{-1}_{TS}.

243

244 2.4.3. Sugar content

245 Reducing sugars of the fermentation substrate were measured following the DNS
246 method (Miller, 1959) in the liquid phase obtained after a solid-liquid extraction using
247 distilled water in a 1:10 (w/v) ratio at 50°C during 30 min. The supernatant was
248 filtered through a 0.45 µm membrane filter and diluted with water in order to obtain a
249 concentration in the range of the calibration curve (Glucose 0.2-2 g L^{-1}).

250

251 2.4.4. C:N ratio, pH, moisture content

252 C:N ratio was determined using a CHNS elemental analyzer Flash 2000 (Thermo
253 Scientific), while moisture content (MC), total solids (TS), volatile solids (VS) and pH
254 were determined according to the standard procedures (The US Department of
255 Agriculture and The US Composting Council, 2001).

256

257 2.5. Statistical analysis

Optimization of the process was carried out using response surface methodology in a Box-Behnken design for 3 factors. 15 experiments were run including 3 replicates in the center point. Analyses were conducted in duplicates. Data was statistically analyzed in Minitab 16 (Minitab Inc.) and the same software was used to find the optimal conditions.

3. Results and discussion

3.1. Precursor effects

Initial experiments consisted of the evaluation of some precursors as additional carbon source to the SCB. Temperature, initial pH, MC and inoculum load were kept at 30°C, 5.3, 76% and 10^8 CFU g^{-1}_{ITS} respectively, based on the results of (Medeiros et al., 2000). Specific air flow rate (S_{AFR}) was set to 0.06 L h^{-1} g^{-1}_{ITS} as suggested by (Medeiros et al., 2001) and air-filled porosity (AFP) of the substrate was estimated according to a method presented by (Agnew et al., 2003) obtaining values in the range 73-78%. Figure 1 shows the OUR profile and cumulative production of volatile compounds of the SSF process when SCB was used alone and after the addition of 10% (dry basis) of glucose and sugar beet molasses, respectively. While glucose has been used as the common precursor for fruit-like aroma production, SBM was used as an alternative carbon source of low price and appropriate characteristics for SSF processes (Jiménez-Peñalver et al., 2016), exploiting its availability giving a second use to this by-product of the sugar industry.

Figure 1.

As it can be observed, the total cumulative volatile production (sum of 15 quantified compounds), was clearly influenced by the dose of precursors. In the reference case, using just SCB as substrate, T_{vol}^{Acc} reached 62.2 mg_{Tvol} g^{-1}_{ITS} while the maximum oxygen uptake rate (approximately 6000 mg_{O2} kg^{-1}_{VS} h^{-1}) took place in a period of 24 h. This result was achieved with an initial reducing sugars content of 26.0% (g_{redSug} g^{-1}_{ITS}). When SCB was supplemented with glucose, initial reducing sugars content reached 38.1%, and this supplementary carbon source increased 70.1% the aroma production until 106.2 mg_{Tvol} g^{-1}_{ITS}. This result is in accordance with the findings of some other authors (Christen et al., 1997; Medeiros et al., 2000; Soares et al., 2000) who have encountered that presence of glucose in the medium is an advantageous condition for promoting the biosynthesis of esters and aldehyde compounds, the main responsible for fruity odor. When SBM was added to the SCB, the production of volatile compounds was also enhanced a 32% respect to SCB alone reaching 82.1 mg_{Tvol} g^{-1}_{ITS}, starting with an equivalent of 27.5% of reducing sugars. This means that the main contribution of SBM is not reducing sugars (around 1.5%) but other carbohydrates. As stated by (Rossi et al., 2009), molasses, in general, also show good characteristics as a co-substrate thanks to the diversity of carbon sources that contains, helping the final substrate to be closer to a natural environment for the microorganism. It was also observed that at these conditions the process is developed faster, reaching a higher OUR_{max} (around 11000 mg_{O2} kg^{-1}_{VS} h^{-1}) in less than 30 h. Although glucose presents a better performance, the use of a pure reagent makes no such attractive the process considering its cost, whereby it is preferable the use of a supplementary carbon source based on by-

products like the sugar beet molasses. Also, we would be dealing with the problem representing its disposal to the environment.

3.2. Moisture content and air flow rate effects

The oxygen availability has been proven to be relevant for the biosynthesis of volatile compounds using different strains (Ito et al., 1990; Medeiros et al., 2001). While a lack of oxygen promotes the production of alcohols and aldehydes through anaerobic routes, a rich oxygen environment makes easier the production of ester compounds, main responsible of fruity aromas. Taking this in mind, the air supply and the moisture content become key parameters in the production of the fruity aromas. To identify the influence of the initial moisture content and the employed air flow rate, a second set of experiments based on a 3^2 factorial design were carried out using as substrate a mixture 1:9 (w/w dry basis) of sugar beet molasses and sugarcane bagasse. For these experiments, the remaining operational conditions were kept identical to those explained in section 3.1 while MC and S_{AFR} have been evaluated from 58% to 77%, (corresponding to a low MC for a typical SSF process and the maximum absorption capacity (MAC) of the substrate) and 0.04 and 0.08 L h^{-1} g^{-1}_{ITS}. Under the experimental conditions assayed, results based on T_{vol}^{Acc}, show that MC had no significant effects on the aroma production (p 0.142) while S_{AFR} (p 0.003) became a relevant factor in the development of the process. This can be observed in Figure 2 (a), where it is clear that the higher the S_{AFR}, the higher the production of volatile compounds regardless of the initial moisture content. There was a trend of higher production in the proximity of MC around 66-68% that can play a major role considering the influence of this variable on other parameters like the

329 substrate porosity and humidification/drying of the media due to its degradation
330 during the process. On the other hand, when results are analyzed from the
331 perspective of ester species production (T_{Ester}^{Acc}), it was found that MC was again
332 not significant (p 0.575) with a slight positive slope indicating a better selectivity
333 towards ester species when working at low MC as detailed in Figure 2 (b).
334 Taking this into consideration, working under low MC would lead to a slower
335 development of the process due to the water availability, but at the same time, it
336 would imply a higher porosity, promoting an aerobic environment. On the other side,
337 working at the MAC (77%) would contribute to the formation of anaerobic zones,
338 which would favor alcohol production, and additionally, the system would be prone
339 to go beyond this absorption capacity given the expected increase in the moisture
340 content due to the mass loss during the fermentation. Considering all these factors,
341 an intermediate point close to 68% of MC seems to be adequate for weighting these
342 effects on the synthesis of the ester species, while favoring the production of total
343 volatile compounds. Moreover, results also show that in the evaluated interval, S_{AFR}
344 was not high enough to produce a dilution effect of the volatile compounds in the air
345 stream due to the increase in the total processed mass of air. This means the
346 headspace concentration of the volatile compounds has no decreased despite the
347 higher amount of air used, which led the process to higher productivities. Therefore,
348 a higher S_{AFR} can be employed without affecting the productivity.
349
350 **Figure 2.**
351
352 3.3. Optimization of the process

353 The production of volatile compounds was optimized through the evaluation of 3
354 operational parameters. First, the air supply, since S_{AFR} plays a major role in the
355 production of the fruity aromas as shown in section 3.2, and from these results is
356 evident that this parameter can be further increased since there are no dilution
357 effects in the upper limit in which it was tested. Furthermore, the temperature was
358 selected considering its importance not only in the *K. marxianus* kinetics but also in
359 the complex mass transfer phenomena involved in the reaction system. Finally, the
360 molasses load was considered as a third factor for the optimization, since it is
361 essential to identify how much co-substrate is it needed for enhancing the
362 productivity as well as the selectivity of the fermentation process. For the
363 experiments, initial pH was fixed in 5.3, MC in 68%, and the inoculum load was 10^8
364 CFU g^{-1}_{ITS}. Table 2 shows the levels used for the experimental design, based on
365 preliminary experiments (data not shown).
366
367 **Table 2.**
368
369 3.3.1. Total cumulative volatile production
370 When T_{vol}^{Acc} was used as the response variable of the optimization, it can be stated
371 that the fermentation process was governed by the temperature. From Figures 3 (a)
372 and (b) it is evident how an increase in temperature was directly linked to an
373 increase in the production of total volatile compounds, reaching the maximum
374 production at 40°C with 161 mgT_{vol} g^{-1}_{ITS}. Also, it was evident the loss of
375 significance of the other two variables when the temperature reaches 30°C or
376 higher, being almost vertical lines in the contour plots of total volatile compounds

production. Equation (3) represents the model for T_{vol}^{Acc} as a function of the evaluated factors with R^2: 98.93%, R^2 (Pred): 83.17% and R^2 (Adj):97.01%.

$$T_{vol}^{Acc} = 161.59 - 10.94T + 607.96S_{AFR} + 0.21mol + 0.20T^2 - 5255.34S_{AFR}^2 - 0.06mol^2 + 6.83TS_{AFR} + 0.04Tmol + 22.51S_{AFR}mol \quad (3)$$

Figure 3.

Working under the conditions maximizing T_{vol}^{Acc} (40°C, S_{AFR} 0.14 L h^{-1} g^{-1}_{ITS}, 35% molasses (dry basis)), the volatile compounds produced during the fermentation were mainly alcohols (43%) of which ethanol was the most abundant (37%), then acetaldehyde (39%) and the esters compounds with a percentage close to 18% (See profile in Figure S2). Given the high alcohol content, it is expected that *K. marxianus* was producing these species preferentially through the anaerobic route rather than the fruity odor components via aerobic routes. In this scenario, some synergistic effects occur thanks to the high temperature level. The high saturation of the hot air supplied has promoted an increase in the MC (Figure 4 (b)), and a faster mass transfer of the products from the solid to the gas phase. Consequently, a loss of weight has also induced the compression of the substrate until AFP between 60-63%, much lower than the initial one.

Figure 4 (a) also shows how the anaerobic fermentation prevails over the aerobic routes when considering the evolution of the OUR and the CO_2^p. From the respiration analysis is evident a higher CO_2 production with respect to the oxygen

401 consumption, particularly in the first 20 h where the respiration quotient
402 ($RQ=CO_2^p/OUR^*(32/44)$) reached values as high as 4 in the first 2 h of processing,
403 followed by 11 h of RQ around 3. This indicates a significant deviation from a pure
404 aerobic process (RQ around 1) as in a composting process (Gea et al., 2004).
405 Furthermore, it is also remarkable the effect of temperature on the *K. marxianus*
406 growth. As observed in Figure 4 (b) there was just a subtle growth during the
407 fermentation even when around 45% of the available reducing sugars were
408 consumed until that point. This behavior could indicate that the yeast was under
409 stressing conditions, and it was forced to metabolize the carbon source into
410 alcohols, instead of using it for its growth. It is during this period that *K. marxianus*
411 seems to be more active, given the rate of consumption of reducing sugars as well
412 as the evolution of the pH, an indirect indicator of the formation of intermediate
413 species during the degradation of the substrate. It was precisely at 24 h when pH
414 has reached its minimum value (4.5) and then a further recovery that coincides with
415 the stabilization of other parameters like the MC, the *K. marxianus* growth and the
416 poor consumption of reducing sugars. Since promoting an aerobic environment is
417 preferable for fruit-like compounds biosynthesis, RQ can be considered as an
418 indirect parameter for monitoring the selectivity to these compounds.
419
420
421 **Figure 4.**
422
423 3.3.2. Fruity odor species - esters production

To identify the operational parameters that favor the production of the ester species, the accumulated sum of only these 9 compounds was also considered as the response variable. As it can be seen in Figure 3 (c), both, temperature and S_{ARF} have and optimum condition that maximizes the ester content in the aroma. This optimum is located close to the mid-range of the variables, around 30°C and 0.11 L h^{-1} g^{-1}_{ITS} producing an accumulated ester production of 47.6 mg_{ester} g^{-1}_{ITS} after 72 h of fermentation. The model representing the response for T_{Ester}^{Acc} is presented in equation 4 with an R^2: 99.04%, R^2 (Pred): 86.27% and R^2 (Adj):97.31%.

$$T_{vol}^{Acc} = -45.17 + 4.18T + 289.72S_{AFR} + 1.13mol - 0.09T^2 - 3198.36S_{AFR}^2 + 0.04mol^2 + 10.02TS_{AFR} + 1.8.10^{-4}Tmol + 5.98S_{AFR}mol \quad (4)$$

Similarly, for the applied molasses load (Figure 3 (d)) the optimum is found at 25%. The marked differences in this scenario respect to the total cumulative volatile production can be better understood analyzing the evolution of the fermentation at these conditions. As Figure 5 (a) states, working at the optimum for producing ester species, promotes an approaching of the OUR and CO_2^p profiles. Consequently, it is expected a lower RQ trend and therefore a less anaerobic environment during the fermentation. In fact, after the first 4 h of processing RQ stabilized around 2, then it has fallen continuously from 18 h to 35 h reaching a value close to 1 which was unchanged until the end of the fermentation. As occurred in all the different runs, produced aroma is a strict function of time, but in this scenario, a pleasant fruity odor was clearly detected. The odor became stronger between the 16 and 30 h of

447 processing, corresponding both to the maximum productivity of volatile compounds
448 as well as the maximum oxygen consumption, similarly to found by other authors
449 (Bramorski et al., 1998b). This effect has occurred thanks to the composition of the
450 produced aroma that contains a total ester contribution of 35% (mainly ethyl
451 acetate) followed by acetaldehyde (27%), ethanol (26%) and other alcohols (12%)
452 (See profile in Figure S3). It is obvious that even in this case there is a significant
453 amount of alcohols in the final aroma, which suggests that parallel metabolization
454 paths compete for the available sugar content. Also, a different picture was
455 presented for the behavior of the *K. marxianus* in the medium. As it can be observed
456 in Figure 5 (b) under these conditions the strain was able to use part of the carbon
457 sources for growing up to 1 order of magnitude, almost depleting the available
458 reducing sugars. Similarly to the findings in section 3.3.1, pH fell at the beginning of
459 the fermentation to a similar level (4.3), but in this case, this drop has matched with
460 the starting of the maximum activity around 18 h. It is also noted that MC has grown
461 at a lower rate than at 40°C, and its stabilization occurred, similarly, after the end of
462 the maximum activity of the *K. marxianus* (42 h).

463

464

465 **Figure 5.**

466

467 3.4. C:N ratio effect

468 The last set of experiments was performed to identify the influence of the nitrogen
469 content in the substrate mixture on the productivity and selectivity of the volatile
470 compounds. As stated in previous studies (Bramorski et al., 1998b; Soares et al.,

2000), C:N ratio directly affects the growing of the strain, and therefore it has a marked influence in the biosynthesis of these secondary metabolites. For this purpose, the C:N ratio was changed from the reference condition using the mixture SCB:SBM (7.5:2.5 w:w) (C:N 58) supplemented with yeast extract until a C:N of 13. These changes were assessed for both scenarios exposed in sections 3.3.1 and 3.3.2 keeping the same operational conditions, and results were expressed per gram of initial substrate added (g_{Isub}). As observed in Figure 6, high nitrogen contents seem to be deleterious for the production of the fruit-like compounds. This trend is specially marked when working at 40°C, where alcohols were the most affected, getting a T_{vol}^{Acc} just of 48% of the value achieved at the reference condition of C:N 58. At 30°C, T_{vol}^{Acc} had no significant changes in the C:N interval from 13 to 39. Under these conditions, it is notable that some other fruity species appeared like Ethyl butanoate, Formic acid pentyl ester or Isoamyl butyrate, but accompanied with pungent compounds like 2-Methyl pyrazine, Crotonaldehyde, Vinyl acetate and Diacetyl. As a consequence, the feeling of the produced aroma has evolved from a pleasant fruity odor to a strong one in the final hours of the fermentation. On the other hand, additional nitrogen has promoted a better growing of the *K. marxianus* both at 30°C and 40°C, reaching a maximum cell count 3.5 times higher than the obtained with the reference substrate (data not shown), agreeing with some author findings (Bramorski et al., 1998a; Soares et al., 2000). These results indicate that similarly to the high temperatures effect, stressing conditions like the scarcity of a nutrient are key factors for promoting the production of the volatile compounds by *K. marxianus.*

Figure 6.

These results show an improvement regarding the productivity for fruity-aromas production. As stated in Table 3, static systems are not able to achieve the same productivity than the continuous systems despite the concentration of the volatile compounds in the headspace of the reactor. This is, continuous systems reach the same, or sometimes higher, concentration levels per hour than the total cumulative concentration of a static system in the headspace. This result is mainly due to the phase equilibrium achieved in static configurations that avoids the further transfer from the solid to the gas phase of the volatile compounds. On the contrary, a continuous air supplied system continuously displaces the equilibrium by stripping the compounds out the system, favoring the further production of these compounds. In addition, compared to other dynamic systems, productivity found in this study is not such far from the best result obtained by (Medeiros et al., 2006), although that system has a bigger scale (1.5 kg substrate), and it uses a rotary drum for improving the interaction between inoculum and substrate. Furthermore, the proposed system reaches these productivities in a short time, saving valuable resources. However, the remarkable aspect lies in the fact these results have been achieved using a substrate composed only of agro-industrial residues and industrial by-products without the need of additional precursors in contrast to previous studies where glucose become an essential component for the metabolization.

519 **Table 3.**

520

521 ## 4. Conclusions

522 *K. marxianus* has proven its efficiency for the biosynthesis of volatile compounds
523 using only agro-industrial residues as sole substrate for the solid-state fermentation.
524 The yeast has also demonstrated its ability to grow in this solid media under very
525 different conditions, using the available nutrients for the production of a variety of
526 aroma compounds according to the operational parameters. The maximum total
527 cumulative volatile production was achieved at 40°C, 0.14 L h^{-1} g^{-1}_{ITS}, 35%
528 molasses (dry basis) with 161 mgT_{vol} g^{-1}_{ITS} producing mostly alcohol species. On the
529 contrary, at 30°C 0.11 L h^{-1} g^{-1}_{ITS}, 25% molasses, the production of ester
530 compounds was maximized (47.6 mg_{ester} g^{-1}_{ITS}), producing a pleasant fruity odor
531 derived from the higher ester species content.
532 Since selectivity to ester species behaves differently than total volatile production,
533 RQ could be used a monitor parameter for promoting the production of these
534 valuable compounds, in further and more sophisticated operating strategies. Using
535 organic residues as nutrient source for SSF show good perspectives for the
536 development of aroma compounds biosynthesis in large-scale processes applying
537 the principle of “from residue to product”.

538

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672 **Table 1.** Volatile compounds identified and quantified during the solid-state

673 fermentation of sugarcane bagasse-sugar beet molasses with *K. marxianus*.

Compound	Odor	Compound	Odor
Aldehydes		**Esters**	
Acetaldehyde	Ripe fruits	Methyl Acetate	Fruity
Crotonaldehyde*	Pungent	Ethyl Acetate	Sweet
Alcohols		Ethyl propionate	Pineapple
Ethanol	-	Propyl Acetate	Pears
2-Propanol	-	Ethyl Isobutyrate	Sweet-Pineapple
Isobutyl Alcohol	Sweet-Musty	Isobutyl Acetate	Raspberry-Pears
Isoamyl Alcohol	Pungent	Isopropyl Acetate	Fruits-Sweet
2-Methyl-1Butanol	Black Truffle	Butyl Acetate	Banana-Apple
Ketones		Isoamyl Acetate	Banana
Acetone*	Sweet	Ethyl Butanoate*	Pineapple

Diacetyl*	Butter	Isoamyl Butyrate*	Pears-Banana

*Identified but not quantified due to the scarce appearance during fermentations.

Table 2. Levels used in the design of experiments for the optimization process.

Factor	Levels		
S_{AFR} (L h^{-1} g^{-1}_{ITS})	0.06	0.10	0.14
T (°C)	21	30	40
mol (%, dry basis)	15	25	35

S_{AFR}: Specific air flow rate; mol: molasses load

Table 3. Main results of several fruity-aroma processes using solid-state fermentation.

Substrate-precursor	Strain	Scale (g_{ITS})-System	Maximum Concentration-Productivity[a]	T_{vol}^{Acc} (µmol L^{-1} g^{-1}_{ITS})[b] (µmol g^{-1}_{ITS})[c]	Time (h)	Ref
SCB-G	*C.F.*	7.5-S	6	307	140	(Christen et al., 1997)
CH-G	*C.F.*	15-S	20	2577	504	(Soares et al., 2000)
CB-G	*K.M.*	10-S	30	3211	72	(Medeiros et al., 2000)
CB-G[c]	*K.M.*	20-D	20	1030	136	(Medeiros et al., 2001)
CH-G[c]	*C.F.*	20-D	23	1315	216	(Medeiros et al., 2006)

CH-G[d]	*C.F.*	525-D	144	4400	192	(Medeiros et al., 2006)
SCB-SBM*	*K.M.*	30-D	105	3494	72	This study
SCB-SBM+	*K.M.*	30-D	70	1808	72	This study

T_{vol}^{Acc}: Total cumulative volatile production; SCB: Sugarcane bagasse; CH.: Coffee husk; G: Glucose; CB: Cassava bagasse; SBM: Sugar beet molasses; C.F.: *C. fimbriata*; *K. marxianus* : *K.M.*; S: Static system; D: Dynamic system; c: Packed bead column; d:Drum reactor; *at 40°C; +: at 30°C. [(a)]For static systems concentration is expressed as µmol of ethanol equivalent L^{-1} g^{-1}_{ITS}, for dynamic systems productivity is expressed as µmol of ethanol equivalent h^{-1} g^{-1}_{ITS}. [(b)]For static systems; [(c)] for dynamic systems.

Figure Captions

Figure 1. Effects of the precursor addition (G: Glucose, SBM: Sugar beet molasses) in the solid-state fermentation of sugarcane bagasse (SCB) with *K. marxianus*. T: 30°C, initial pH: 5.3, MC: 76%, S_{AFR}: 0.06 L h^{-1} g^{-1}_{ITS}.

Figure 2. Moisture content and air flow rate effects (a) on the total cumulative volatile production, and (b) on the cumulative ester species production for the solid-state fermentation of sugarcane bagasse (SCB) with *K. marxianus*. T: 30°C, initial pH: 5.3, 10% molasses (dry basis).

Figure 3. Effects of air flow rate and molasses addition as a function of temperature during the solid-state fermentation of sugarcane bagasse with *K. marxianus*. (a), (b) effects on the total cumulative volatile production, (c), (d) effects on the cumulative ester species production. Initial pH: 5.3, MC: 68%.

Figure 4. Behavior of the solid-state fermentation along time at 40°C: (a) OUR, CO_2^p and total volatile production; (b) pH, MC, cells count and reducing sugars

727 profiles. T: 40°C, initial pH 5.3, MC: 68%, S_{AFR} 0.14 L h^{-1} g^{-1}_{ITS}, 35% molasses (dry
728 basis).

729 **Figure 5.** Behavior of the solid-state fermentation along time at 30°C: (a) OUR,
730 CO_2^p and total volatile production; (b) pH, MC, cells count and reducing sugars
731 profiles. T: 30°C, initial pH 5.3, MC: 68%, S_{AFR} 0.11 L h^{-1} g^{-1}_{ITS}, 25% molasses (dry
732 basis).

733 **Figure 6.** Effect of C:N ratio on the production of the cumulative total volatiles and
734 cumulative ester species.

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737 **Figure 1.**

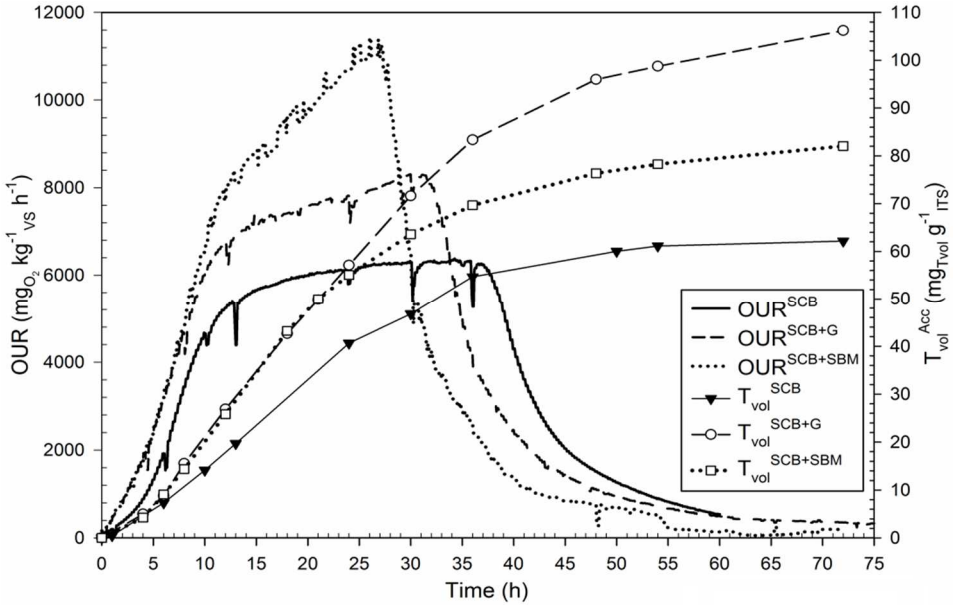

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Figure 2.

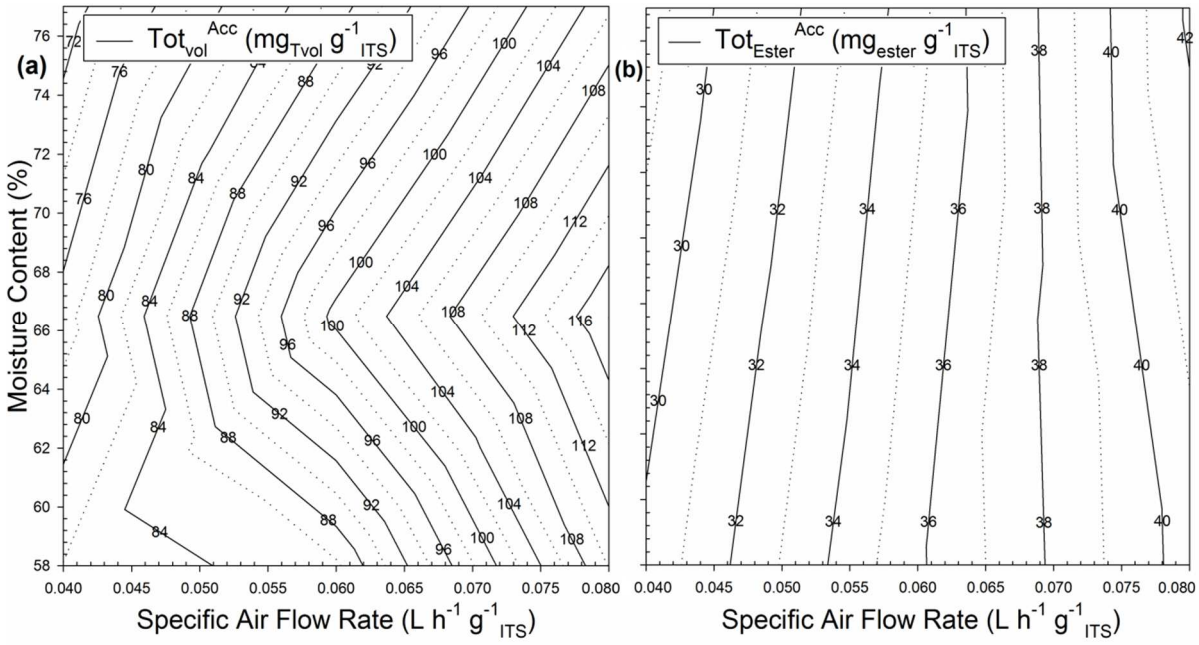
(a)
(b)
Moisture Content (%)
Specific Air Flow Rate (L h-1 g-1 ITS)
Specific Air Flow Rate (L h-1 g-1 ITS)

Figure 3.

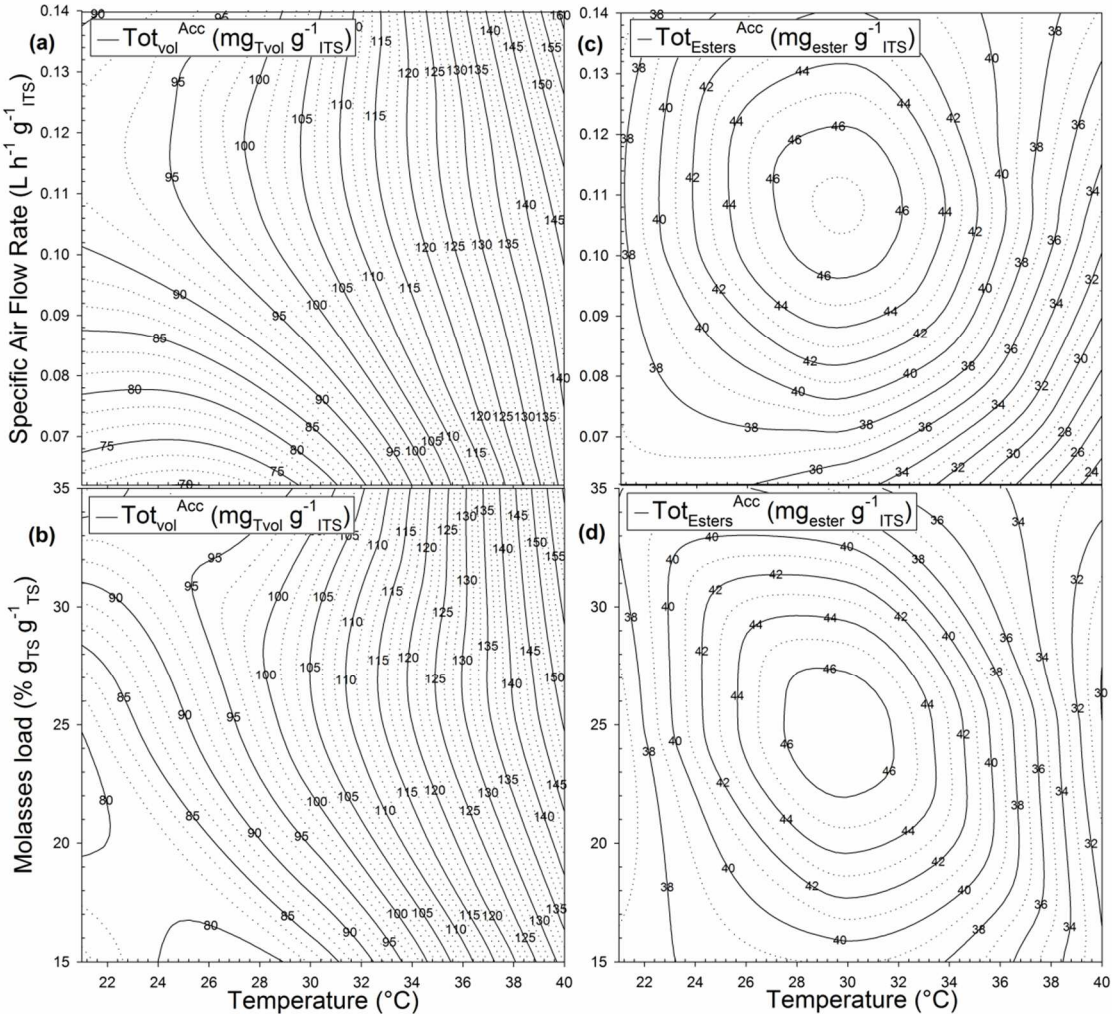
(a)
(b)
(c)
(d)
Specific Air Flow Rate (L h-1 g-1 ITS)
Molasses load (% gTS g-1 TS)
Temperature (°C)
Temperature (°C)

Figure 4.

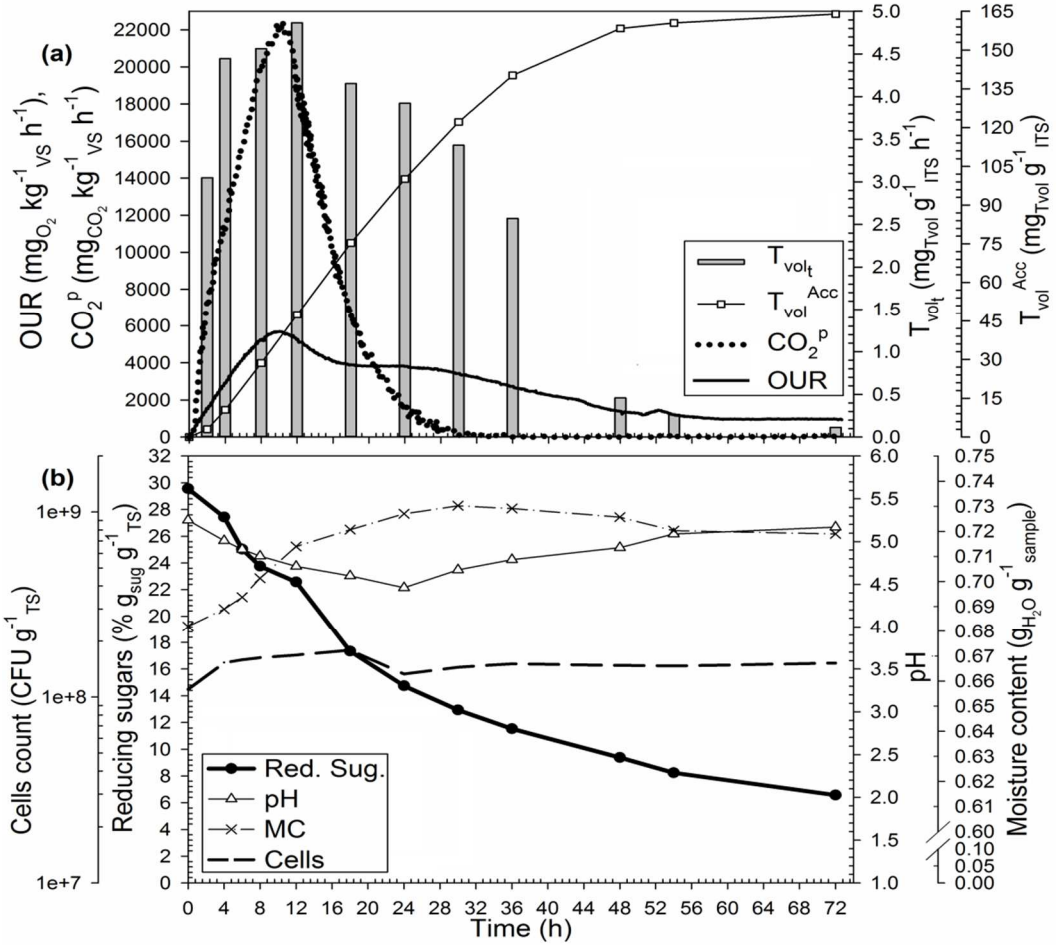
(a)
OUR (mg_{O_2} kg^{-1}_{VS} h^{-1}), CO_2^p (mg_{CO_2} kg^{-1}_{VS} h^{-1})
T_{vol_t} (mg_{Tvol} g^{-1}_{ITS} h^{-1})
T_{vol}^{Acc} (mg_{Tvol} g^{-1}_{ITS})
T_{vol_t}
T_{vol}^{Acc}
CO_2^p
OUR
(b)
Cells count (CFU g^{-1}_{TS})
Reducing sugars (% g_{sug} g^{-1}_{TS})
pH
Moisture content (g_{H_2O} g^{-1}_{sample})
Red. Sug.
pH
MC
Cells
Time (h)

Figure 5.

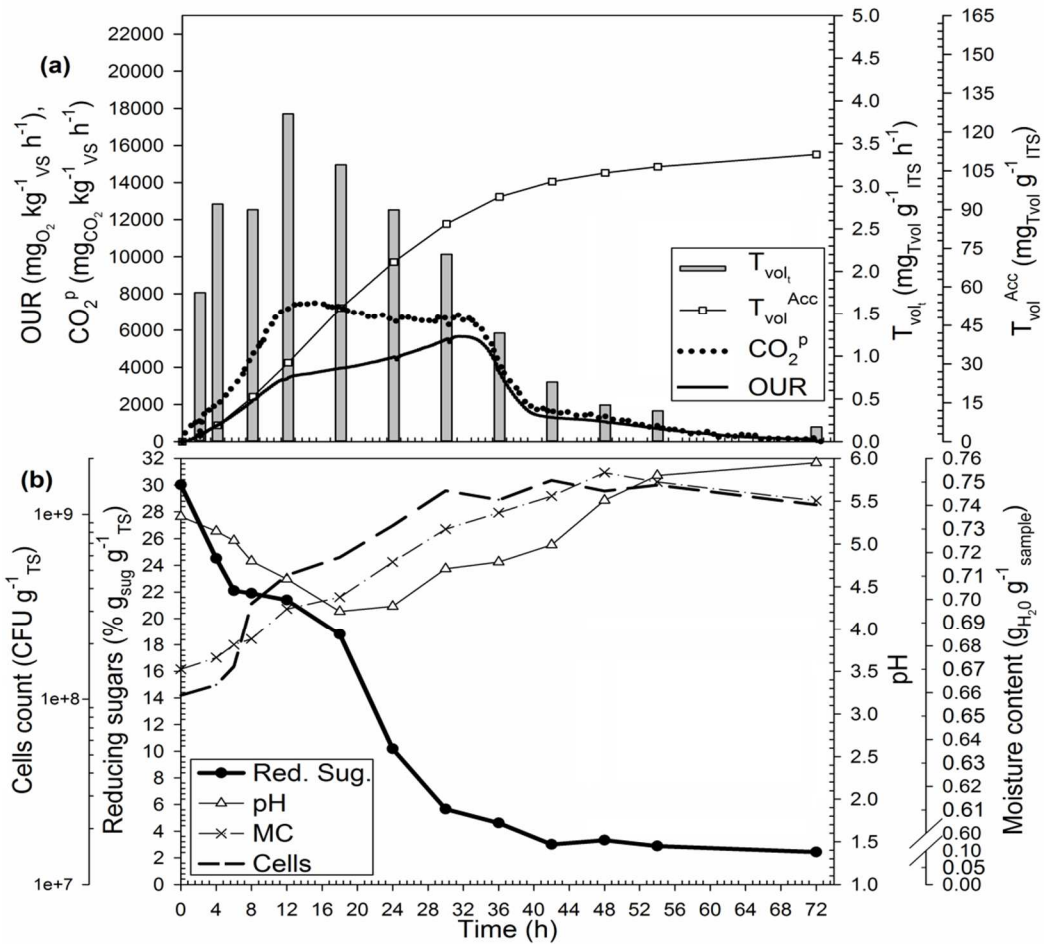
(a)
OUR (mg_{O_2} kg^{-1}_{VS} h^{-1}), CO_2^p (mg_{CO_2} kg^{-1}_{VS} h^{-1})
T_{vol_t} (mg_{Tvol} g^{-1}_{ITS} h^{-1})
T_{vol}^{Acc} (mg_{Tvol} g^{-1}_{ITS})
T_{vol_t}
T_{vol}^{Acc}
CO_2^p
OUR
(b)
Cells count (CFU g^{-1}_{TS})
Reducing sugars (% g_{sug} g^{-1}_{TS})
pH
Moisture content (g_{H_2O} g^{-1}_{sample})
Red. Sug.
pH
MC
Cells
Time (h)

Figure 6.

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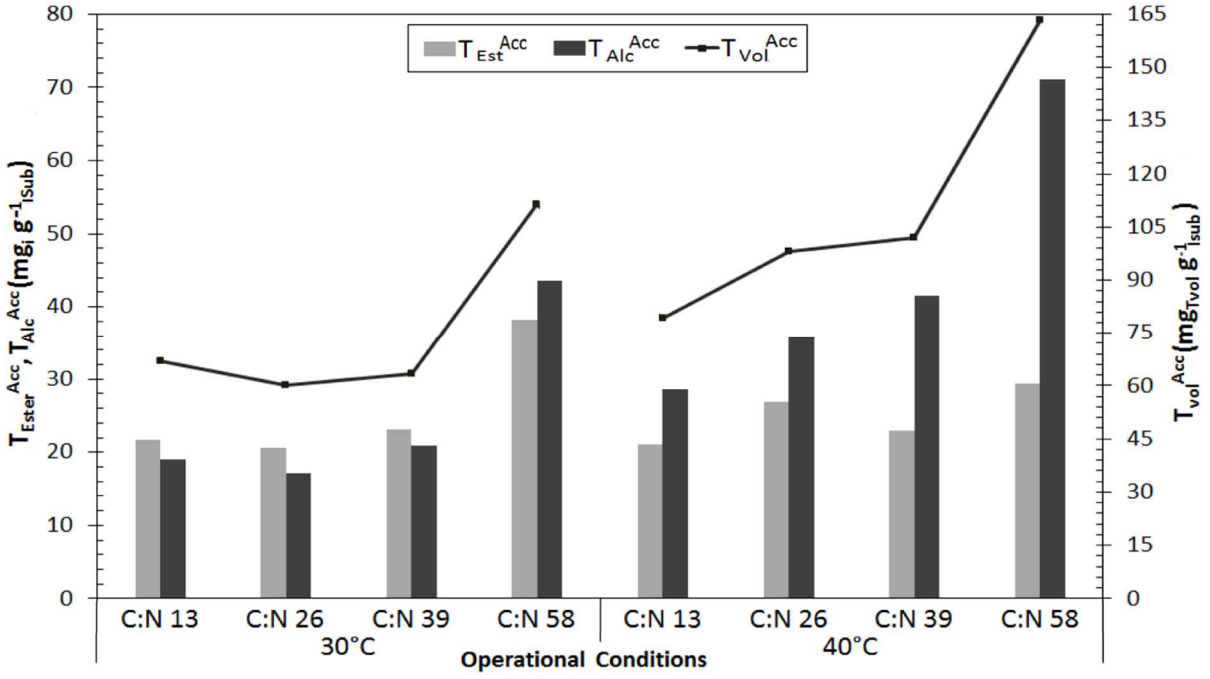
T_{Est}^{Acc}
T_{Alc}^{Acc}
T_{Vol}^{Acc}
T_{Ester}^{Acc}, T_{Alc}^{Acc} ($mg_i\ g^{-1}_{Isub}$)
T_{vol}^{Acc} ($mg_{Tvol}\ g^{-1}_{Isub}$)
0
10
20
30
40
50
60
70
80
0
15
30
45
60
75
90
105
120
135
150
165
C:N 13
C:N 26
C:N 39
C:N 58
C:N 13
C:N 26
C:N 39
C:N 58
30°C
40°C
Operational Conditions